

Scotland's Rural College

## **Long live the liver: immunohistochemical and stereological study of hepatocytes, liver sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells of male and female rats throughout ageing**

Marcos, R; Correia-Gomes, C

*Published in:*  
Cell and Tissue Research

*DOI:*  
[10.1007/s00441-016-2490-y](https://doi.org/10.1007/s00441-016-2490-y)

First published: 05/09/2016

*Document Version*  
Peer reviewed version

[Link to publication](#)

### *Citation for pulished version (APA):*

Marcos, R., & Correia-Gomes, C. (2016). Long live the liver: immunohistochemical and stereological study of hepatocytes, liver sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells of male and female rats throughout ageing. *Cell and Tissue Research*, 366(3), 639 - 649. <https://doi.org/10.1007/s00441-016-2490-y>

### **General rights**

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

### **Take down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1 Long live the liver: immunohistochemical and stereological study of  
2 hepatocytes, liver sinusoidal endothelial cells, Kupffer cells and hepatic  
3 stellate cells of male and female rats throughout ageing

4  
5 Ricardo Marcos<sup>1</sup>, Carla Correia-Gomes<sup>2</sup>  
6

7 <sup>1</sup> Laboratory of Histology and Embryology, Department of Microscopy, Institute of Biomedical  
8 Sciences Abel Salazar, University of Porto, ICBAS-UP, Portugal

9 <sup>2</sup> Scotland's Rural College (SRUC), Epidemiology Research Unit – Future Farming Systems  
10 Group, Inverness, United Kingdom.  
11  
12

13 Running title: Male and female differences in the liver throughout ageing.  
14

15 Keywords: Hepatic stellate cells, Kupffer cells, hepatocytes, liver, ageing, stereology.  
16

17 Corresponding author: Ricardo Marcos (DVM, MD, PhD)

18 Laboratory of Histology and Embryology  
19 Institute of Biomedical Sciences Abel Salazar  
20 Rua de Jorge Viterbo Ferreira no. 228,  
21 4050-313 Porto, Portugal  
22 Email: [rmarcos@icbas.up.pt](mailto:rmarcos@icbas.up.pt)  
23

## **Abstract**

It has been known that both male versus female differences in liver enzyme activity and gene expression in the liver are attenuated with ageing. Nevertheless, the effect of ageing on liver structure and quantitative cell morphology remains unknown. Male and female Wistar rats of two, six, 12 and 18 months were examined. Stereological techniques and immunohistochemical tagging of Hepatocytes (HEP), liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC) and hepatic stellate cells (HSC) were applied to assess the total number and number per gram of these cells throughout ageing. The mean cell volume of HEP and HSC, lobular position and liver collagen content were also evaluated with stereological techniques. The number per gram of HSC was similar for both genders and maintained throughout ageing. The mean volume of HSC was also conserved, but differences in the cell body and lobular location were observed. As to HEP, statistically significant gender differences were noted in young rats (females had smaller and more binucleated HEP) but were attenuated with ageing. The same occurred for KC and LSEC, since the higher number per gram in young females disappeared in older animals. As to liver collagen, it increased with ageing, but only in males. Herein, we highlighted that the numbers of these four cell types were related throughout ageing, with well-defined cell ratios. The shape and lobular position of HSC changed with ageing in both males and females. As to HEP, KC and LSEC, the gender dimorphism of the young rat liver disappeared with ageing.

## Introduction

Nowadays, it is widely agreed that perfusion is reduced in the aged liver (Schmucker and Sanchez 2011; Loustaud-Ratti et al. 2016). This affects, for instance, transhepatocellular transport of dyes and IgA (Popper 1985), diffusion of small lipoproteins (Hilmer et al. 2005), bile salt formation (Le Couteur and McLean 1998; Vollmar et al. 2002) and the clearance of drugs. Differences in microsomes have been reported with ageing, with a decrease in cytochrome-P-450 concentration and NADPH-cytochrome C reductase activity (Van Bezooijen 1984; Popper 1985; Frith et al. 2009). Notably, for rodents, this occurs mainly in the male liver, because enzyme levels in females remain fairly unchanged with ageing (Kitani 1992). In effect, a sort of feminization of the male liver occurs with ageing, as enzymes more active in younger males usually decline to approach the activity levels seen in the female liver (and those less active in males increase up to the female levels) (Kitani 1992; 2007). Curiously, this pattern also appears to exist at the level of gene expression (Kwekel et al. 2010). Nevertheless, ageing effects on liver structure are much less clear. The state of the art in this field is characterised by few consistent observations and a lack of correlation between structural and functional data (Zeeh 2001). Furthermore, to the best of our knowledge, the liver structure of the aged male and female liver has never been studied in detail by quantitative morphology.

Despite this, the last decade has shed some light on liver ageing. It has been reported that sinusoids in the aged liver have fewer *fenestrae*, surrounded by basal lamina and collagen, leading to so-called pseudocapillarisation (Le Couteur et al. 2001; 2008). Furthermore, intralobular collagen was reported to increase with ageing (Gagliano et al. 2002). Since this collagen is mainly produced by hepatic stellate cells (HSC), at least in pathological conditions, it could be hypothesized that morphological differences would appear with ageing. Only a few studies on liver ageing have explored the HSC role on liver ageing, either in a qualitative (Enzan et al. 1991) or in a semi-quantitative perspective (Martin et al. 1992; Imai et al. 2000; Vollmar et al. 2002; Warren et al. 2011) and their conclusions were controversial. Moreover, studying gender ageing differences among rats is quite relevant due to the common practice of using males and females interchangeably for experimental studies, as well as *in vitro* protocols. For instance, older rats — typically male and/or retired female breeders (Ramadori and Saille 2002; Tacke and Weiskirchen 2005; Friedman 2008) — are recommended for use

when isolating HSC; however, it is unknown whether HSC differ in quantitative morphology between genders.

In order to reveal eventual ageing differences a quantitative approach should be applied (e.g. using stereology), since the qualitative morphology alone may overlook important structural changes. Other methods, such as cell isolation, are unable to reveal liver cell ratios, since the cell yield from these methods varies between parenchymal and non-parenchymal cells. Nevertheless, an in-depth knowledge of liver structure (and cell ratios) is important for the bioengineering construction of artificial livers and for *in vitro* studies, since precise cell ratios are necessary to model paracrine effector mechanisms in co-culture models. Studies *in vitro* have used ratios of parenchymal and non-parenchymal cells varying from 10:1 to 1:10 have been used (Bathia et al. 1999), which may not mirror the *in vivo* organization of the aged liver. Furthermore, it has been stressed that the true aspects of ageing are difficult to ascertain from a simple comparison between young and old animals (since changes can occur in between), or from the study of only the male gender (Kitani 1992; Schmucker 2001).

In this study we used design-based stereology methods to study morphological changes of the rat liver throughout ageing in both males and females. We looked for differences in the size of lobules, collagen content and in the total number (N) and number per gram (N/g) of hepatocytes (HEP), Kupffer cells (KC), HSC and liver sinusoidal endothelial cells (LSEC). Finally, we studied the cell volume as well as the position of HSC in liver lobules.

## Materials and Methods

### *Animals*

We used male and female Wistar rats (n = 5 per group) of two months old, adult (six months old), middle-aged (12 months old) and old (18 months old), initially bought from Charles-River Laboratories (Barcelona, Spain). When considering the mean lifespan of this strain, these ages correspond to around 10%, 25%, 50% and 75% of their lifespan respectively (Porta et al. 1980; Sawada and Carlson 1987, Manikonda and Jagota 2012). All the animals had been weaned at 20 days and were kept in standard conditions, receiving water and food (Mucedola® 4RF21, Settimo Milanese, Italy) *ad libitum*. The rats were housed in pairs or individually (old males) in a controlled environment [temperature (25°C) and 12 hours light-dark cycle]. Animal management followed European Union Directives (1999/575/CE and 2010/63/UE) for the protection

of animals used for scientific purposes and the study was approved by local ethical authorities (ORBEA ICBAS-UP Project 152/2016).

### ***Tissue Preparation***

Sampling was performed during the morning period (from 10:00 to 12:00), to circumvent oscillations in liver function due to circadian rhythmicity (Davidson et al. 2004). In females, daily vaginal cytologies were observed, in order to avoid collecting samples in proestrous/oestrous days. Beforehand, animals were deeply anaesthetised with ketamine plus xylazine. Blood was collected and centrifuged to obtain serum for assessing alanine transaminase and aspartate transaminase levels. Transcardiac perfusion was performed with an isosmotic solution; the liver was weighed and its volume determined by the Scherle's method, as detailed elsewhere (Marcos et al. 2012). A smooth fractionator sampling scheme was applied (Marcos et al. 2012): half of the paraffin blocks was used for thick sections (30  $\mu\text{m}$  thick) and exhaustively sectioned, whilst the other half was used for thin sections (3  $\mu\text{m}$  thick). Of the thick sections, five sections in every 30 were sampled in order to immunostain against: 1) glial fibrillary acidic protein for estimating the N and N/g of HSC; 2) ED2 for estimating the N and N/g of KC; 3) E-cadherin, to differentiate mononucleate HEP from binucleated HEP, estimating their percentage, and to assess the N and N/g of HEP; 4) Von Willebrand Factor to estimate the N and N/g of LSEC; 5) glial fibrillary acidic protein and glutamine synthetase [an established marker of centrilobular HEP (Gebhardt and Mecke 1983)] to evaluate the lobular distribution of HSC. Thin sections were used for immunostaining against: 1)  $\alpha$ -smooth muscle actin to evaluate the existence of activated HSC; 2) glial fibrillary acidic protein to determine the relative volume of HSC (whole cell); 3) glutamine synthetase, to estimate the lobular size, by measuring the porto-central distance. Thin sections were also used for assessing liver collagen, by Sirius red staining. In addition, tiny liver fragments ( $< 0.5 \text{ mm}^3$ ) were removed from the rat liver for electron microscopy. These were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH = 7.4) for two hours and subsequently post-fixed in phosphate buffered 1%  $\text{OsO}_4$  for another two hours. After dehydration in ethanol and propylene oxide, the pieces were embedded in epoxy resin. Semithin sections were obtained and stained with methylene blue-azur II, which were used to quantify volume densities of HSC (cell body). Additionally, ultrathin sections were obtained, contrasted with uranyl acetate and lead citrate and observed in a transmission electron microscope, JEOL 100CXII, at 60 kV.

### ***Immunohistochemistry***

The protocol used for thick sections has been previously described (Marcos et al. 2004; 2006; Santos et al. 2009). Briefly, antigen recovery was carried out in microwave [four plus four minutes, at 600 W, in buffered citrate 0.01M (pH = 6.0)] and a streptavidin–biotin protocol was used (Histostain Plus, Invitrogen, Camarillo, California) for all antibodies, except for LSEC [pre-treatment of tissues with pepsin (Sigma, St. Luis, Missouri) 0.4% in HCl 0.01M for 30 minutes]. For glial fibrillary acidic protein, we used 1:3000 rabbit polyclonal antibody (Dako, Glostrup, Denmark), whereas for ED2 and E-cadherin we used monoclonal mouse antibodies, from Serotec (Oxford, United Kingdom) diluted at 1:100 and from Dako (clone NCH 38) diluted at 1:250, respectively. It is opportune to mention that ED2 is unanimously recognized as a marker of fully differentiated, long-lived KC (Roskams et al. 2007; Santos et al. 2009). Additionally, we used an antibody against Von Willebrand Factor from Dako, diluted at 1:3200, in order to tag LSEC. All the slides were incubated for four days at 4° C.

For the double immunohistochemistry, slides were also placed in the microwave (this time for three cycles of four minutes at 600W in buffered citrate). After blocking endogenous biotin and peroxidase, the first streptavidin–biotin protocol was followed with antibody against glial fibrillary acidic protein (1:1500 dilution for four days at 4°C). Slides were developed for two minutes in 0.05% 3,3'-diaminobenzidine (Dako) in Tris-buffered saline with 0.03% H<sub>2</sub>O<sub>2</sub>. Sections were then rinsed in tap-water and dipped in 50 mM glycine buffer (pH = 2.2) for five minutes, to strip off the antibodies of the first immunoreaction. The second streptavidin–biotin protocol against glutamine synthetase followed, using 1:4000 rabbit polyclonal antibody (graciously provided by Professor Rolf Gebhardt, University of Leipzig), for another four days at 4°C. Slides were developed with aminoethylcarbazole (Dako) for 10 to 20 minutes (final red colour controlled by microscopic observation) and mounted in Aquatex (Dako).

To quantify glial fibrillary acidic protein in thin sections, a streptavidin–biotin protocol was also used (Histostain Plus), but with lower dilutions (1:1200) and overnight incubation. The blocking solution, the secondary antibody and the streptavidin–peroxidase complex were all applied for 20 minutes and colour development in diaminobenzidine was restricted to two minutes. As for  $\alpha$ -smooth muscle actin

immunostaining, slides were placed in a pressure cooker for three minutes in citrate buffer (pH = 6.0). After rinsing in phosphate buffered saline, a polymer based immunohistochemical protocol was followed [Novocastra Novolink Polymer (Leica Biosystems, Newcastle, United Kingdom)]. The protein blocking solution was applied for five minutes and  $\alpha$ -smooth muscle actin antibody (clone HM45, Dako) diluted at 1:500 immersed the slides overnight. The post-primary solution and polymer were both applied for 30 minutes. Finally, slides were developed for two minutes in diaminobenzidine. For assessing the porto-central distance, immunohistochemistry against glutamine synthetase was used (diluted at 1:4000), following the protocol for glial fibrillary acidic protein immunostaining in thin sections.

Positive and negative controls (omission of first antibody and replacement by non-immune serum) were included, both in thin and thick sections, and all slides were evaluated blindly (*i.e.*, the observer was unaware of the gender or age of the animal), to avoid eventual observer-related bias.

#### ***Histochemistry with Sirius-red***

Five thin sections were randomly selected per animal, de-waxed and hydrated. The counterstaining was achieved with celestial blue and haematoxylin, each for 5 minutes. After washing in tap water, the Sirius red (Sigma, coloration index 35782) dissolved in picric acid (1 mg/ml) was applied for 1 hour at room temperature (Kumar 2005). After washing in acidified water (1% acetic acid), the sections were dehydrated in ethanol, cleared in xylene and mounted in DPX.

#### ***Morphometrical Analysis***

In the thin sections, the linear distance between a central venule (surrounded by HEP immunostained against glutamine synthetase) and the closest portal tract was measured using the CAST-Grid software (version 1.5, Olympus). Both peripheral (closer to Glisson's capsule) and inner lobules were considered for the measurements.

#### ***Stereological Analysis***

A stereology workstation with CAST-Grid software was used (Marcos et al. 2012). At the monitor, a final magnification of 4750x allowed easy and accurate recognition of all cells. Throughout the disector height (20  $\mu\text{m}$ ), a software generated counting frame with defined areas (1673  $\mu\text{m}^2$ , 1267  $\mu\text{m}^2$  and 418  $\mu\text{m}^2$  for HSC, KC/LSEC and HEP,



respectively) was used for unbiased counting. For assessing the position of HSC in liver lobules, a systematic uniform random sampling was applied, but HSC were counted only if fields were in the vicinity of the portal tracts or central venules (5-6 HEP around those landmarks were settled upon as boundaries). For the purpose of counting cells, their nucleus was considered the counting unit (in the case of binucleated HEP, this was predetermined to be the first nucleus in focus), and cells were counted following optical disector rules (Marcos et al. 2012). The N of cells in the whole liver was estimated using optical fractionator formulae (Marcos et al. 2004; 2012). Simultaneously, the N/g was determined, as it is useful for comparing animals with different liver weights. The coefficient of error of the number of cells counted was also estimated (Marcos et al. 2004; 2012).

Additionally, the mean cell volume, so-called number-weighted mean cell volume, of mononucleate and binucleated HEP was estimated by the nucleator method (Gundersen 1988; Marcos et al. 2012). In the latter, the first nucleus with nucleolus in focus was considered for the measurements (Marcos et al. 2012). In the case of HEP with two nucleoli (or more), two (or more) measurements were performed (Gundersen 1988).

Semithin and glial fibrillary acidic protein immunostained sections were used, respectively, in order to estimate the relative volume of the cell body and whole cell of HSC. In both cases, point counting (grid with 108 points) was used to estimate the relative volume (Figure 1). The number-weighted mean volume was then obtained by an indirect approach, through the division of the relative volume by the relative number of HSC per unit of volume (corrected for paraffin shrinkage) (Marcos et al. 2012). As to the assessment of collagen content, point-counting (grid with 36 points) was also performed in the CAST-Grid software. In a preliminary study, we have shown that this test-system allowed an easy discrimination of collagen fibres (Marcos et al. 2015).

### ***Statistical analysis***

The normality of the data was checked using the Shapiro-Wilk's test. Pearson's correlation analysis was performed to detect linear correlations. After checking the homogeneity of variances (Levene's test) a two-way ANOVA was performed taking into consideration the effects of gender and ageing. When significant differences existed, multiple comparisons were done using the post-hoc Tukey's test. Statistical significance level was set at  $p \leq 0.05$ . The software SPSS 18 (IBM, Armonk, United

States of America) was used. Quantitative results are presented with their mean and standard deviation.

## Results

### *Qualitative findings*

All livers displayed a normal morphology, without noticeable differences across animals at optical and electron microscopy. A consistent and reliable marking of HEP was achieved with E-cadherin, allowing a clear distinction between mononucleate and binucleated HEP. For glial fibrillary acidic protein, HSC were immunostained in both periportal and centrilobular areas (Supplemental figure 1). The HSC of aged rats had larger and more numerous lipid droplets than young rats; these cells occasionally protruded into sinusoids (Figure 1). However, no differences were noticeable between HSC of males and females. No staining of HSC with  $\alpha$ -smooth muscle actin antibody was observed (Supplemental figure 2). Fully differentiated KC also exhibited a stellate appearance with ED2, but with shorter and much thicker cytoplasmic processes. Regarding LSEC, they had a characteristic dark nucleus layered on a thin rim of immunomarking against Von Willebrand Factor (Figure 2).

### *Quantitative findings*

The livers of males were heavier ( $p < 0.001$ ) than those of females ( $15.15 \pm 1.6$  g *versus*  $9.62 \pm 1.2$  g, respectively). The liver-to-body weight ratio was highest at the age of two months ( $4.27\% \pm 0.7\%$ ,  $p < 0.01$ ) compared to other age groups ( $2.72\% \pm 0.3\%$ ); no gender differences were observed. A strong correlation was observed between liver and body weight ( $r = 0.77$ ,  $p < 0.001$ ). Hepatic transaminases values were within the reference ranges, presenting no statistically significant differences ( $43.1 \pm 4.0$  IU/L and  $39.1 \pm 9.3$  IU/L for alanine transaminase, and  $101.4 \pm 17.6$  IU/L and  $105.2 \pm 14.5$  IU/L for aspartate transaminase in males and females, respectively).

An average of 62 lobules per rat was analysed by morphometry. The average porto-central distance was  $450 \pm 17$   $\mu$ m in males and  $412 \pm 22$   $\mu$ m in females (data corrected for paraffin shrinkage). No statistically significant differences were observed for the size of lobules throughout ageing and between genders (Table 1).

An average of 216 fields per animal was screened to assess liver collagen. The amount of intralobular collagen corresponded to 56% in males and 46% in females, without significant differences with ageing. This collagen was moderately correlated with the N of HSC ( $r = 0.50$ ;  $p < 0.01$ ) and with the N of HEP ( $r = 0.47$ ;  $p < 0.01$ ). Liver collagen was influenced by gender ( $p < 0.001$ ) and ageing ( $p < 0.01$ ), namely in males. In fact, collagen was more abundant in males than in females in adult and old rats (Figure 3). This was due to intralobular collagen, since the collagen around central veins and in portal tracts was maintained throughout ageing and gender (varying between 0.9 and 1.3% of liver collagen).

From each rat, between 291 and 780 optical disectors were analysed to obtain the N and N/g of HSC, HEP, KC and LSEC; and between 150 and 216 fields per rat were assessed to determine the relative volume of HSC (cell body and whole cell). The N of HSC was higher in males ( $209 \pm 14 \times 10^6$ ) than in females ( $154 \pm 15 \times 10^6$ ) ( $p = 0.016$ ), but the N/g was similar across genders ( $\approx 14.6 \pm 1.3 \times 10^6$ ) (Figure 4). Neither of these parameters was associated with ageing. A correlation was observed between the N of HSC and *i*) liver weight ( $r = 0.85$ ,  $p < 0.01$ ), *ii*) N of HEP ( $r = 0.73$ ;  $p < 0.0001$ ) and *iii*) with the N of KC ( $r = 0.53$ ,  $p < 0.01$ ). Due to the increased volume of lipid droplets present in older HSC, the number-weighted mean volume of the cell body increased with ageing ( $144 \pm 61 \mu\text{m}^3$  to  $576 \pm 104 \mu\text{m}^3$ ) ( $p < 0.01$ ), but the volume of the whole cell was relatively stable, varying from  $593 \pm 134 \mu\text{m}^3$  to  $796 \pm 192 \mu\text{m}^3$  (Figure 5). The distribution of HSC was not influenced by gender; however, an ageing pattern was observed ( $p < 0.001$ ): in younger animals HSC were more frequently located pericentrally ( $56.5 \pm 4.9\%$ ) whereas in older animals these cells were more abundant in a periportal location ( $61.2 \pm 6.7\%$ ).

The N of HEP did not vary with ageing ( $\approx 2.0 \pm 0.3 \times 10^9$ ), in contrast with the N/g of HEP ( $159 \pm 33 \times 10^6$  in young and  $184 \pm 26 \times 10^6$  in old) ( $p < 0.05$ ) (Figure 6). The N of HEP was statistically correlated with *i*) body and *ii*) liver weights ( $r = 0.60$ ;  $p < 0.0001$ , for both) and *iii*) with the N of KC ( $r = 0.5$ ,  $p < 0.01$ ), whereas the N/g of HEP was correlated with the N/g of KC and N/g of binucleated HEP ( $r = 0.94$ ,  $p < 0.001$  and  $r = 0.75$ ,  $p = 0.02$ , respectively). Gender differences were observed in the N/g of HEP, but these were restricted to younger animals ( $136 \pm 11 \times 10^6$  in males and  $183 \pm 39 \times 10^6$  in females) ( $p = 0.016$ ). Similarly, gender differences existed for binucleated HEP, as well as their percentage, which were higher in females ( $25 \pm 4\%$  in young males and  $34 \pm 5\%$  in young females), but these differences were attenuated with ageing ( $27 \pm 5\%$

and  $31 \pm 4\%$  in old males and females, respectively) (Figure 6). The percentage of binucleated HEP was negatively correlated with the body weight ( $r = -0.81$ ,  $p = 0.015$ ). For the number-weighted mean cell volume no statistically significant difference was observed with ageing and gender [except between mononuclear HEP in young males and females ( $5861 \pm 369 \mu\text{m}^3$  and  $4915 \pm 293 \mu\text{m}^3$ , respectively) ( $p < 0.01$ )] (Figure 7). In average, the volume of binucleated HEP was  $7177 \pm 752 \mu\text{m}^3$ , being 31 to 59% greater than that of mononucleate HEP ( $p < 0.001$ ).

The N of fully differentiated KC was moderately correlated with liver weight ( $r = 0.67$ ,  $p < 0.001$ ). Overall, this parameter was stable with ageing ( $\approx 286 \pm 58 \times 10^6$ ), as was the N/g of KC ( $\approx 23 \pm 3 \times 10^6$ ); however, gender differences were observed in young animals ( $19 \pm 3 \times 10^6$  and  $30 \pm 6 \times 10^6$  in males and females, respectively) ( $p = 0.016$ ) (Figure 8). Likewise, the N of LSEC was also stable with ageing and gender ( $\approx 802 \pm 25 \times 10^6$ ). Nevertheless, gender differences existed for the N/g ( $p < 0.001$ ) and these occurred mostly in young animals ( $40 \pm 4 \times 10^6$  and  $95 \pm 11 \times 10^6$  in males and females, respectively) ( $p < 0.0001$ ) (Figure 9).

By estimating the N of HEP, HSC, KC and LSEC in the same set of animals, it was possible to estimate the ratios among these cell types in the male and female rat liver (Figure 10). Overall, the percentage of HEP, LSEC, KC and HSC in the rat liver was 60%, 21%, 8.9% and 5.8%, respectively in males, and 56%, 25%, 7.8% and 4.8%, respectively in females.

It is noteworthy that the coefficient of error for estimations of cell number (N) were always below the recommended threshold of 10% (Marcos et al. 2012), being comprised of between 3.9% and 6.8%. Therefore, the sampling procedure was responsible for up to 24% of the total observed variance — *i.e.*, the variance due to the methodological procedures was much less important than the biological variability.

## Discussion

To the best of our knowledge, this is the first study of liver ageing using quantitative morphology that addressed differences in both males and females. Wistar rats were used in this study because, on one hand, this is one of the most common stock of animals used in liver research, and on the other hand, this strain has few age-related liver lesions — the only lesion consistently reported are altered cell foci, which tend to occur at a later age, around 2.5 years (Van Bezooijen 1984). Wistar rats therefore differ from other

strains used in gerontology research, such as Fischer or Sprague-Dawley rats. Fischer strains have altered cell foci at an earlier age than Wistar, and may have focal chronic hepatitis and bile duct hyperplasia (Van Bezooijen 1984). Sprague-Dawley rats have been described as exhibiting periportal inflammation, sinusoidal enlargement, fatty change and eosinophilic foci on reaching older ages (Van Bezooijen 1984; Sakai et al. 1997). As to sporadic tumors, aged Wistar rats have an increased incidence of pituitary adeno(carcino)ma, mammary adeno(carcino)ma (in females) and Leydig cell tumor (in males), but hepatocellular neoplasms are reported to be rare (Eiben and Bomhard 1999). The major drawback of using the Wistar strain on liver ageing studies, however, is that animals have to be aged in-house, since suppliers have a limited number of available rats (females over six months cannot be bought from existing companies).

Overall, this study highlighted statistically significant correlations between the numbers of different liver cell types in the rat liver. This is a new finding that supports the existence of a morphofunctional organisation, with well-defined ratios of parenchymal and non-parenchymal cells maintained throughout ageing (Figure 10). These ratios have been previously hypothesized by Rojkind et al. (2011) but have never been determined throughout ageing in males and females. Considering the volume estimation of mononucleate and binucleated HEP and their relative abundance, a theoretical porto-central cell cord composed of  $17 \pm 1$  HEP in males and  $16 \pm 1$  HEP in females was estimated. Another major conclusion that can be drawn from this study is that the ageing process attenuated most of the cytological differences in liver cells. The gender dimorphism that existed in young animals for HEP, KC and LSEC, but not for HSC, disappeared at older ages. Such a trend has never been revealed by morphology, but it actually follows known patterns for enzymatic activities (Kitani 2007) and gene expression (Kwekel et al. 2010). An exhaustive micro-array gene expression study with rats highlighted that most gene differences decreased by middle-age and were completely abolished in old animals (Kwekel et al. 2010).

Our initial hypothesis that morphological differences for HSC could exist with ageing was not supported by our data, since no differences were observed in the number or mean volume of HSC. So far only HSC “numbers per area” have been determined along ageing, however the studies reached to controversial conclusions. According to Cogger and Le Couteur (2009) and Warren et al. (2011), HSC doubled their number with ageing. Whereas Enzan et al. (1991) reported no significant differences and Vollmar et al. (2002) suggested that the number of HSC could decrease up to 30% with ageing.

However, it should be acknowledged that two-dimensional counts (“numbers per area”) are biased and may not reveal the three-dimensional reality of biological tissues (Mandarim-Lacerda 2003; Marcos et al. 2012). The larger cell body of an aged HSC could be prone to an overestimation bias in two-dimensional counts. The shift from a centrilobular to a periportal predominance of the HSC with ageing is another new finding. It has been reported that quiescent HSC are able to slowly move through the space of Disse (Senoo et al. 2007; Friedman 2008), being dynamic in the changeable 3D structure of the sinusoids (Sato et al. 2003; Senoo et al. 2007). It has long been known that the matrix composition differs along the porto-central axis (Reid et al. 1992; Roskams et al. 2007; Senoo et al. 2010). It is conceivable that an increase of factors that drive HSC migration, *e.g.* transforming growth factor- $\beta$ 1 (Yang et al. 2003), could occur with ageing. It is noteworthy that aged HSC were not activated, being  $\alpha$ -smooth muscle actin antibody negative, as previously described (Warren et al. 2011). However, their shape was different with ageing. Classical and more recent studies have reported that HSC in aged animals appear swollen, having significantly more and larger lipid droplets than in young animals (Enzan et al. 1991; Warren et al. 2011). This was confirmed by us, since a significant increase in the number-weighted mean volume of the cell body occurred with ageing. Based on such differences, some authors concluded that aged HSC are larger than young HSC (Vollmar et al. 2002; Schmucker 2005); however our study suggests that, based on the number-weighted mean volume of HSC, the cells actually change from a small cell body with long and thin extensions in youngsters to a large cell body with thicker and much shorter extensions in older rats. Actually, this large cell body justifies the recommendation of using old animals for isolating HSC (Ramadori and Saille 2002; Tacke and Weiskirchen 2005; Friedman 2008) — since cells contain more lipid droplets they can be better separated by gradient centrifugation. Moreover, the larger cell body of HSC should also contribute to the blood flow reduction in sinusoids of older animals (Vollmar et al. 2002; Warren et al. 2011) because it protrudes into sinusoids — as has previously been reported (Warren et al. 2011) — and HSC shift to periportal areas. It may be hypothesized that the shorter processes of aged HSC should surround and control the blood flow of fewer sinusoids than in youngsters, in whom HSC encircle more than two sinusoids (Friedman 2008). Whether or not hyperplasia and/or hypertrophy of HEP occur with ageing remain controversial questions. The porto-central distance estimated in this study is in

accordance with previous reports for rats (Wagenaar et al. 1994; Ruijter et al. 2004; Warren et al. 2008) and no ageing differences in this axis were found. In the ageing process it is thought that only enlargement of previously existing lobules occurs (Vollmar et al. 2002). However, considering that the N/g of HEP increases by 35% between young and old males and the porto-central axis is maintained, two hypotheses may be placed: lobules grow in height and/or new lobules could be formed. Taking into account that the relative volume of collagen within portal tracts and central venules was maintained throughout ageing, an increase in height (*i.e.*, taller lobules) would probably be more important. This increased height, bearing an increased number of primary classical lobules at the base (liver surface), would justify the greater number of superficial lobules reported in the rat (Papp et al. 2009).

Another controversy surrounding HEP relates to the increase of binucleated HEP with ageing (Wheatley 1972, Schmucker 1998). It is unanimously agreed that polyploidy increases in the aged liver and some authors also reported an upsurge in binuclearity (Popper 1985; Schmucker 1998; Malarkey et al. 2005). Even so, in our set of rats, we did not find statistically significant ageing differences in the N of binucleated HEP or in their percentage. This is in agreement with previous studies, based on different methodologies in mouse and rat (Epstein 1967; Wheatley 1972; Faggioli et al. 2011). It is noteworthy that the percentage of binucleated HEP was negatively correlated with body and liver weight. This has already been reported in the rat elsewhere (Vinogradov et al. 2001), with very similar figures ( $r \approx -0.57$ ).

The N of fully differentiated KC has never been evaluated throughout ageing, to the best of our knowledge. Hilmer et al. (2007) reported a 3-fold increase in “numbers per area” of KC with ageing, using thin paraffin sections stained by haematoxylin-eosin. Technical concerns may explain the differences to our study, such as the use of haematoxylin-eosin, which is undesirable for quantification due to uncontrolled bias in the counts, but also size differences (larger KC in old animals) may lead to overestimations when using “numbers per area”. Other studies have suggested that the volume of KC increases with ageing (Martin et al. 1992; 1994), probably due to an accumulation of non-functional material in cytoplasm (Martin et al. 1994).

Classical and more recent studies have addressed the structure of LSEC throughout ageing. While the reduction in the number of *fenestrae* (ageing defenestration) in LSEC is nowadays well documented in different species (Le Couteur et al. 2001; 2008; Cogger and Le Couteur 2009), the N and N/g have been sparsely detailed. Still, it was

proposed that the number and percentage of LSEC were constant throughout ageing (De Leeuw et al. 1990), and it was reported that numbers of sinusoids remained fairly unchanged during the lifespan of rats (Vollmar et al. 2002), which is consistent with our findings. It has been determined that N/g of LSEC was around  $20 \times 10^6$  (De Leeuw et al. 1990) and that these cells comprise 20% of all liver cells (Malarkey et al. 2005). According to our findings, the N/g of LSEC is higher ( $49 \times 10^6$  and  $79 \times 10^6$  in males and females respectively) and these cells comprise 21% to 26% of all liver cells.

The liver has much less collagen than most other organs — only  $\approx 5\%$  (Roskams et al. 2007; Friedman 2008), as we observed herein. Previous studies have followed the collagen content throughout ageing, but only in males, and noted that it increased with ageing (Porta et al. 1981; Gagliano et al. 2002). Curiously, both studies documented a collagen peak in 6 months' animals, as we observed. The reason for such an increase in collagen is unknown, but it may traduce a remodelling activity with formation of new lobules, which is believed to occur during liver growth (Roskams et al. 2007). At that age, as well as in older animals, it has been shown that the metalloproteinase activity was significantly reduced (Gagliano et al. 2002). It should be emphasised that (intralobular) collagen cannot be solely related to HSC, since the expression of collagen I has been attributed to LSEC, HSC and HEP (Roskams et al. 2007). In this vein, we observed a significant correlation between collagen content and two latter cell types, suggesting that both HSC and HEP may be important for collagen synthesis in the normal organ.

In conclusion, this study contributes to the state of the art about the process of liver ageing, highlighting that the liver structure is well preserved. Herein, we provided defined ratios between cells, which are relevant for bioengineering construction of artificial livers and for *in vitro* studies, namely using co-culture models. Except for collagen content, the male/female differences are attenuated by ageing and, in this vein, no major cytological or structural changes in normal liver cells should be able to compromise the long life of the liver.

## References

Bathia SN, Balis UJ, Yarmush ML, Toner M (1999) Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *FASEB J* 13, 1883-1990.



481 Eiben R, Bomhard EM (1999) Trends in mortality, body weights and tumor incidences  
 482 of Wistar rats over 20 years. *Experimental Toxicological Pathology* 51, 523-536.  
 483 Cogger VC, Le Couteur DG (2009) Fenestrations in the liver sinusoidal endothelial cell.  
 484 In: *The liver: biology and pathobiology*, 5th edition (Arias IM, Alter HJ, Boyer JL,  
 485 Cohen DE, Fausto N, Shafritz DA, Wolkoff AW eds), pp 389-406. John Wiley & Sons  
 486 Ltd.  
 487 Davidson AJ, Castañón-Cervantes O, Stephan FK (2004) Daily oscillations in liver  
 488 function: diurnal vs circadian rhythmicity. *Liver International* 24, 179-86.  
 489 De Leeuw AM, Brouwer A, Knook DL (1990) Sinusoidal endothelial cells of the liver:  
 490 fine structure and function in relation to age. *Journal of Electron Microscopy Technique*  
 491 14:218-236.  
 492 Enzan H, Saibara T, Ueda H, Onishi S, Yamamoto Y, Okada T, Hara H (1991)  
 493 Ultrastructural observation of Ito cells in the aged rats. In: *Cells of the Hepatic Sinusoid*  
 494 (Wisse E, Knook DL, McCuskey RS eds), pp. 226–229. Kupffer Cell Foundation.  
 495 Epstein CJ (1967) Cell size, nuclear content, and the development of polyploidy in the  
 496 Mammalian liver. *Proceedings of the National Academy of Sciences of the United*  
 497 *States of America* 57, 327–334.  
 498 Faggioli F, Vezzoni P, Montagna C (2011) Single-cell analysis of ploidy and  
 499 centrosomes underscores the peculiarity of normal hepatocytes. *PLoS One* 6, e26080.  
 500 Friedman SL (2008) Hepatic stellate cells: protean, multifunctional, and enigmatic cells  
 501 of the liver. *Physiological Reviews* 88, 125-172.  
 502 Frith J, Jones D, Newton JL (2009) Chronic liver disease in an ageing population. *Age*  
 503 *and Ageing* 38, 11-18.  
 504 Gagliano N, Arosio B, Grizzi F, Masson S, Tagliabue J, Dioguardi N, Vergani C,  
 505 Annoni G (2002) Reduced collagenolytic activity of matrix metalloproteinases and  
 506 development of liver fibrosis in the aging rat. *Mechanisms of Ageing and Development*  
 507 123, 413-425.  
 508 Gebhardt R, Mecke D (1983) Heterogeneous distribution of glutamine synthetase  
 509 among rat liver parenchymal cells in situ and in primary culture. *The EMBO Journal* 2,  
 510 567-570.  
 511 Gundersen HJ (1988) The nucleator. *Journal of Microscopy* 151, 3-21.  
 512 Hilmer SN, Cogger VC, Le Couteur DG (2007) Basal activity of Kupffer cells increases  
 513 with old age. *The Journals of Gerontology. Series A, Biological Sciences and Medical*  
 514 *Sciences* 62, 973-978.

515 Imai K, Sato M, Kojima N, Miura M, Sato T, Sugiyama T, Enomoto K, Senoo H (2000)  
 516 Storage of lipid droplets in and production of extracellular matrix by hepatic stellate  
 517 cells (vitamin A-storing cells) in Long-Evans cinnamon-like colored (LEC) rats.  
 518 *Anatomical Record* 258, 338-348.  
 519 Kitani K (1992) Liver and aging. *Gastroenterologia Japonica* 27, 276-285.  
 520 Kitani K (2007) What really declines with age? The Hayflick Lecture for 2006 35th  
 521 American Aging Association. *Age* 29, 1-14.  
 522 Kwekel JC, Desai VG, Moland CL, Branham WS, Fuscoe JC (2010) Age and sex  
 523 dependent changes in the liver gene expression during the life cycle of the rat. *BMC*  
 524 *Genomics* 11, 675.  
 525 Kumar RK (2005) Morphological methods for assessment of fibrosis. In: *Fibrosis*  
 526 *Research, Methods and Protocols* (Varga J, Brenner DA, Phan SH eds), pp. 179-207.  
 527 Humana Press.  
 528 Le Couteur DG, McLean AJ (1998) The aging liver. Drug clearance and an oxygen  
 529 diffusion barrier hypothesis. *Clinical Pharmacokinetics* 34, 359-373.  
 530 Le Couteur DG, Cogger VC, Markus AM, Harvey PJ, Yin ZL, Anselin AD, McLean  
 531 AJ (2001) Pseudocapillarization and associated energy limitation in the aged rat liver.  
 532 *Hepatology* 33, 537-543.  
 533 Le Couteur DG, Warren A, Cogger VC, Smedsrød B, Sørensen KK, De Cabo R, Fraser  
 534 R, McCuskey RS (2008) Old age and the hepatic sinusoid. *The Anatomical Record* 291,  
 535 672-683.  
 536 Loustaud-Ratti V, Jacques J, Debette-Gratien M, Carrier P (2016) Hepatitis B and  
 537 elders: an underestimated issue. *Hepatology Research* 46, 22-28.  
 538 Malarkey DE, Johnson K, Ryan L, Boorman G, Maronpot RR (2005) New insights into  
 539 functional aspects of liver morphology. *Toxicologic Pathology* 33, 27–34.  
 540 Mandarim-de-Lacerda CA (2003) Stereological tools in biomedical research. *Anais da*  
 541 *Academia Brasileira de Ciências* 75, 469-486.  
 542 Manikonda PK, Jagota A (2012) Melatonin administration differentially affects age-  
 543 induced alterations in daily rhythms of lipid peroxidation and oxidant enzymes in male  
 544 rat liver. *Biogerontology* 13, 511-524.  
 545 Marcos R, Monteiro RAF, Rocha E (2004) Estimation of the number of stellate cells in  
 546 a liver with the smooth fractionator. *Journal of Microscopy* 215, 174-182.  
 547 Marcos R, Monteiro RAF, Rocha E (2006) Design-based stereological estimation of  
 548 hepatocyte number, by combining the smooth optical fractionator and

immunocytochemistry with anticarcinoembryonic antigen polyclonal antibodies. *Liver International* 26, 116-124.

Marcos R, Monteiro RAF, Rocha E (2012) The use of design-based stereology to evaluate volumes and numbers in the liver: a review with practical guidelines. *Journal of Anatomy* 220, 303-317.

Marcos R, Bragança B, Fontes-Sousa AP (2015) Image analysis or stereology: which to choose for quantifying fibrosis? *Journal of Histochemistry and Cytochemistry* 63, 734-736.

Martin G, Sewell RB, Yeomans ND, Morgan DJ, Smallwood RA (1994) Hepatic Kupffer cell function: the efficiency of uptake and intracellular degradation of <sup>14</sup>C-labelled mitochondria is reduced in aged rats. *Mechanisms of Ageing and Development* 73, 157-168.

Martin G, Sewell RB, Yeomans ND, Smallwood RA (1992) Ageing has no effect on the volume density of hepatocytes, reticulo-endothelial cells or the extracellular space in livers of female Sprague-Dawley rats. *Clinical and Experimental Pharmacology & Physiology* 19, 537-539.

Papp V, Dezső K, László V, Nagy P, Paku S (2009) Architectural changes during regenerative and ontogenic liver growth in the rat. *Liver Transplantation* 15, 177-183.

Popper H (1985) Coming of age. *Hepatology* 5, 1224-1226.

Porta E, Joun N, Nitta R (1980) Effects of the type on dietary fat at two levels of vitamin E in Wistar male rats during development and aging. I. Life span, serum biochemical parameters and pathological changes. *Mechanisms of Ageing and Development* 13, 1-39.

Porta E, Keopuhiwa L, Joun N, Nitta R (1981) Effects of the type on dietary fat at two levels of vitamin E in Wistar male rats during development and aging. III. Biochemical and morphometric parameters of the liver. *Mechanisms of Ageing and Development* 15, 297-335.

Ramadori G, Saile B (2002) Mesenchymal cells in the liver - one cell or two? *Liver* 22, 283-294.

Reid LM, Fiorino AS, Sigal SH, Brill S, Holst PA (1992) Extracellular matrix gradients in the space of Disse: relevance to liver biology. *Hepatology* 15, 1198-1203.

Rojkind M, Philips GG, Diehl AE (2011) Microarchitecture of the liver: a jigsaw puzzle. *Journal of Hepatology* 54, 187-188.

582 Roskams T, Desmet VJ, Verslype C (2007) Development, structure and function of the  
583 liver. In: MacSween's pathology of the liver, 5th edition (Burt A, Portmann B, Ferrell L  
584 eds), pp. 1-73. Churchill Livingstone.

585 Ruijter JM, Gieling RG, Markman MM, Hagoort J, Lamers WH (2004) Stereological  
586 measurement of porto-central gradients in gene expression in mouse liver. *Hepatology*  
587 39, 343-352.

588 Sakai Y, Zhong RR, Garcia B, Zhu L, Wall WJ (1997) Assessment of the longevity of  
589 the liver using a rat transplant model. *Hepatology* 25, 421-425.

590 Santos M, Marcos R, Santos N, Malhão F, Monteiro RAF, Rocha E (2009) An unbiased  
591 stereological study on subpopulations of rat liver macrophages and on their numerical  
592 relation with the hepatocytes and stellate cells. *Journal of Anatomy* 214, 744-751.

593 Sato M, Suzuki S, Senoo H (2003) Hepatic stellate cells: unique characteristics in cell  
594 biology and phenotype. *Cell Structure and Function* 28, 105-112.

595 Sawada M, Carlson J (1987) Changes in the superoxide radical and lipid peroxide  
596 formation in the brain, heart and liver during the lifetime of the rat. *Mechanisms of*  
597 *Ageing and Development* 41, 125-137.

598 Schmucker DL (1998) Aging and the liver: an update. *Journal of Gerontology* 53, 315-  
599 320.

600 Schmucker DL (2001) Liver function and phase I drug metabolism in the elderly: a  
601 paradox. *Drugs Aging* 18, 837-851.

602 Schmucker DL (2005) Age-related changes in liver structure and function: Implications  
603 for disease? *Experimental Gerontology* 40, 650-659.

604 Schmucker DL, Sanchez H (2011) Liver regeneration and aging: a current perspective.  
605 *Current Gerontology and Geriatric Research* 2011, 526379.

606 Senoo H, Kojima N, Sato M (2007) Vitamin A-storing cells (stellate cells). *Vitamins*  
607 *and Hormones* 75, 131-159.

608 Senoo H, Yoshikawa K, Morii M, Miura M, Imai K, Mezaki Y (2010) Hepatic stellate  
609 cell (vitamin A-storing cell) and its relative — past, present and future. *Cell Biology*  
610 *International* 34, 1247-1272.

611 Tacke F, Weiskirchen R (2012) Update on hepatic stellate cells: pathogenic role in liver  
612 fibrosis and novel isolation techniques. *Expert Review of Gastroenterology &*  
613 *Hepatology* 6, 67-80.

Van Bezooijen CFA (1984) Influence of age-related changes in rodent liver morphology and physiology on drug metabolism - a review. *Mechanisms of Ageing and Development* 25, 1-22.

Vinogradov AE, Anatskaya OV, Kudryavtsev BN (2001) Relationship of hepatocyte ploidy levels with body size and growth rate in mammals. *Genome* 44, 350-360.

Vollmar B, Pradarutti S, Richter S, Menger MD (2002) In vivo quantification of ageing changes in the rat liver from early juvenile to senescent life. *Liver* 22, 330-341.

Wagenaar GT, Moorman AF, Chamuleau RA, Deutz NE, De Gier C, De Boer PA, Verbeek FJ, Lamers WH (1994) Vascular branching pattern and zonation of gene expression in the mammalian liver. A comparative study in rat, mouse, cynomolgus monkey, and pig. *The Anatomical Record* 239, 441-452.

Warren A, Chaberek S, Ostrowski K, Cogger VC, Hilmer SN, McCuskey RS, Fraser R, Le Couteur DG (2008) Effects of old age on vascular complexity and dispersion of the hepatic sinusoidal network. *Microcirculation* 15, 191-202.

Warren A, Cogger VC, Fraser R, DeLeve LD, McCuskey RS, LeCouteur DG (2011) The effects of old age on hepatic stellate cells. *Current Gerontology and Geriatrics Research* 2011, 439835.

Wheatley DN (1972) Binucleation in the mammalian liver. *Experimental and Cell Research* 74, 455-465.

Yang C, Zeisberg M, Mosterman B, Sudhakar A, Yerramalla U, Holthaus K, Xu L, Eng F, Afdhal N, Kalluri R (2013) Liver fibrosis: insights into migration of Hepatic stellate cells in response to extracellular matrix and growth factors. *Gastroenterology* 124, 147-159.

Zeeh J (2001) The aging liver: consequences for drug treatment in old age. *Archives of Gerontology and Geriatrics* 32, 255-263.

Figure 1 – Semithin section of liver parenchyma of an old rat. A hepatic stellate cell (arrows), engorged with lipid droplets, protrudes into a sinusoid. For illustrative purposes, the counting grid (composed of 108 points) used for estimating the relative volume of the cell body of hepatic stellate cells is shown; in this case, two points (red) would be counted.

Figure 2 – Thick liver sections (30  $\mu\text{m}$ ) immunostained against: (A) E-cadherin to highlight the borders of mono- and binucleated hepatocytes; (B) ED-2 to tag fully differentiated Kupffer cells; (C) Von Willebrand factor to depict liver sinusoidal endothelial cells and (D) glial fibrillary acidic protein to mark hepatic stellate cells. The counting grid is shown for illustrative purposes, being larger (D) for less abundant cells and smaller for the most numerous (A). Cells were counted if their nucleus was inside the counting grid or touched the inclusion lines (green), but not the exclusion ones (red). The cells counted in this example are pointed with arrowheads; bar = 9  $\mu\text{m}$ .

Figure 3 – Relative volume of liver collagen in male and female young, adult, middle-aged and old rats. Data expressed as mean  $\pm$  standard deviation; statistically significant differences between genders (\*) and to young animals ( $\Psi$ ).

Figure 4 – Total number (N) and number per gram (N/g) of hepatic stellate cells in male and female young, adult, middle-aged and old rats. Data expressed as mean  $\pm$  standard deviation; (\*) statistically significant differences between genders.

Figure 5 – Number-weighted mean volume of hepatic stellate cells in male and female young, adult, middle-aged and old rats. Full lines refer to the volume of whole cell and dotted lines to the cell body. Data expressed as mean  $\pm$  standard deviation; ( $\Psi$ ,  $\gamma$ ) statistically significant differences to old animals.

Figure 6 – Total number (N) and number per gram (N/g) of hepatocytes (mononucleate and binucleated) and of binucleated hepatocytes in male and female young, adult, middle-aged and old rats. Data expressed as mean  $\pm$  standard deviation; statistically significant differences between genders (\*) and age groups ( $\gamma$ ).

Figure 7 – Number-weighted mean volume of mononucleate and binucleated hepatocytes in male and female young, adult, middle-aged and old rats. Data expressed as mean  $\pm$  standard deviation; (\*) statistically significant differences between genders.

Figure 8 – Total number (N) and number per gram (N/g) of hepatocytes in male and female young, adult, middle-aged and old rats. Data expressed as mean  $\pm$  standard deviation; (\*) statistically significant differences between genders.

Figure 9 - Total number (N) and number per gram (N/g) of liver sinusoidal endothelial cells in male and female young, adult, middle-aged and old rats. Data expressed as mean  $\pm$  standard deviation; (\*) statistically significant differences between genders.

Figure 10 – Estimated cell ratios of hepatocytes, Kupffer cells, hepatic stellate cells and liver sinusoidal endothelial cells in male and female rats (considering all age groups).

### **Supplemental figures**

Supplemental Figure 1 - Thick section of an adult male liver immunostained against glial fibrillary acidic protein. Hepatic stellate cells (arrows) can be seen around the portal tract (P) and central veins (V). For unequivocal identification of these, glutamine synthetase immunomarking (that tags pericentral hepatocytes) was also used.

Supplemental Figure 2 - Thin section of old male (A) and old female (B) rat liver immunostained against  $\alpha$ -smooth muscle actin. Immunomarking is restricted to the wall of blood vessels in portal tracts (A) and in the central vein (B), without noticeable positive cells alongside the sinusoids; bar = 30  $\mu$ m.